Changes in transcription profile and cytoskeleton morphology in pelvic ligament fibroblasts in response to stretch: the effects of estradiol and levormeloxifene

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Failure of ligamentous support of the genital tract to resist intra-abdominal pressure is a plausible underlying mechanism for the development of pelvic organ prolapse, but the nature of the molecular response of pelvic tissue support remains unknown. We hypothesized that the expression of genes coding for proteins involved in maintaining the cellular and extracellular integrity would be altered as a result of mechanical stretch. Therefore, cDNA microarrays were used to examine the difference in transcriptional profile in RNA of primary culture fibroblasts subjected to mechanical stretch and those that remained static. Out of 34 mechano-responsive genes identified (P < 0.05), four were coding for regulation of actin cytoskeleton remodelling, and its interaction with the extracellular matrix proteins; these are phosphatidyl inositol-4-phosphate 5-kinase (PIP5K1C), the human signal-induced proliferation associated gene-1 (SIPA-1), TNFRSF1A-associated via death domain (TRADD) and deoxyribonuclease 1-like 1 (DNase 1-L1). The transcriptosomal changes led us to investigate the phenotypic consequences of stretch, levormeloxifene and estradiol (E2) on the cytoskeleton of cultured fibroblasts. The percentage of cells with abnormal F-actin configuration was significantly higher in fibroblasts subjected to stretch compared with the static model (P < 0.0001). Levormeloxifene caused similar significant alterations in actin morphology of the static fibroblasts. The use of E2 did not reverse the process or protect the cells from the effect of stretch, but significantly increased the rate of fibroblast proliferation, suggestive of a role in healing process. Mechanical stretch and/or levormeloxifene disturb the fibroblasts ability to maintain the cytoskeleton architecture and we speculate that they may disrupt ligamentous integrity and result in clinical prolapse.

Keywords: actin; levormeloxifene; stretch; microarray; prolapse

Introduction

Pelvic organ prolapse (POP) is a common condition affecting ~15% of parous women, and is most frequently seen in the post-menopausal state (Mokrzycki et al., 1997). The exact mechanism behind POP remains poorly understood, however, it is likely that repeated stretch throughout life, accentuated during pregnancy and childbirth, plays a significant role, as do changing hormonal influences.

Our group has shown that there is a change in the constituents of the extracellular matrix (ECM) in uterine cardinal ligament tissue obtained from patients with POP. These changes were partly reversed in patients who had received hormone replacement therapy. It is unlikely that POP is simply a product of hypo-oestrogenemia. The discontinuation of two selective estrogen receptor modulators (SERMs) (idoxifen and levormeloxifene) was related to the increased incidence of POP and urinary incontinence (Novo-Nordisk, 1998b; Hendrix and McNeeley, 2001). This is strongly suggestive that different estrogen receptors (ER) mediate different effects on the ECM. Indeed, we have previously reported clearly discernible raised levels of expression of ER-α, progesterone receptor and androgen receptor in the cardinal ligaments of the prolapsed uteri when compared with those with no prolapse (Ewies et al., 2004). A number of in vitro studies on human fibroblasts have demonstrated that mechanical stretch can modulate cell behaviour through several different signalling pathways. Stretching in vitro fibroblasts induced cell proliferation (Lambert et al., 1992; Liu et al., 1992; Zeichen et al., 2000; Barkhausen et al., 2003), influenced differentiation towards the myofibroblast phenotype through the induction of α-smooth muscle actin-containing stress fibres (Tomasek et al., 1992), and changed their morphology (Lambert et al., 1992). Mechanical stretch applied to knee ligaments fibroblasts in culture altered collagen I and III mRNA expression (Hsieh et al., 2000), induced nitric oxide production (van Griensven et al., 2003) and enhanced secretion of interleukin-6 (Skutek et al., 2001). Similarly, stretch induced a significant increase in collagen α1 mRNA and fibronectin protein synthesis by vascular smooth muscle cells (Stanley et al., 2000), while in scleral fibroblasts, it increased production of tissue inhibitor of metalloproteinase-1 (Yamaoka et al., 2001). A number of different methods of stretch as well as different tissues were used in these experiments. This suggests that ECM responses to stretch are either specific to the tissue or to the mechanism of stretch, or more likely, to a combination of both.
We propose that excessive stretch of the uterine cardinal ligaments is an important factor in the development of genital prolapse by changing cellular function. The aim of this study is to show that a previously validated method of stretch applied to fibroblasts derived from healthy pelvic ligaments will induce changes in the transcriptional profile that can inform hypotheses regarding the mechanism of POP. Further, the identification of a number of stretch-responsive genes involved in the regulation of actin cytoskeleton remodelling led us to test the hypotheses that the mechanical stretch of these fibroblasts would alter the F-actin morphology, and that the application of estradiol (E2) and levomefloxetine would have significant, and different, effects.

Materials and Methods

Source of fibroblasts

Samples of uterine cardinal ligament tissue were obtained from abdominal hysterectomy procedures performed on premenopausal Caucasian women with heavy periods with no malignancy or history of uterine prolapse. Tissue removal was performed with Local Ethics Committee Approval and patient informed consent.

Primary culture

The specimens were placed immediately in a cold Hanks’ Balanced Salt Solution, HBSS, (Gibco, Paisley, Scotland, UK) and minced into 1 mm3 cubes, treated with 1% Collagenase type I (Gibco) in HBSS at 37 °C for 2 h with gentle rotation. The supernatant, containing the fibroblasts, was washed with HBSS and Medium 199 Earle’s MOD Salts (Gibco) and 15% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA). The cell suspension was spun down twice to remove collagenase. The pellets were re-suspended in growth medium, and plated in 25 cm2 tissue culture flasks with Nunclon surface (Nalge Nunc International Corporation, Herford, UK). The medium was changed on alternate days and confluent fibroblasts cells were passaged into a 75 cm2 flask (Nalge Nunc International Corporation) using 0.1% trypsin-EDTA (Gibco) for 10 min at 37°C.

Stretcher experiments

Preparation of cells for treatment

Fibroblasts from the fourth to sixth passages were seeded (5 × 104 cells/well) into 6-well BioFlex® flexible plates with Collagen-I coated elastomer membrane bottoms (FlexCell International Corporation, McKeesport, PA, USA), and allowed to grow. They were serum-starved for 48 h by using 1% charcoal/dextran treated heat-inactivated fetal bovine serum added to the tissue culture medium to achieve quiescence, and then they were cultured with 15% of the same serum added to the tissue culture medium.

Chronic cyclical mechanical stretch

These experiments were performed in the Flexercell Stress Unit (FlexCell International Corporation) (Hsieh et al., 2000; Stanley et al., 2000). We developed the stretch model such that maximum stretch for a maximum duration is exerted to the limits endured by the elastomer membrane. We found that 21% stretch, in 12 h cycles for a duration of 96 h proved to be the limit of the apparatus beyond which these membranes started to leak. The corresponding BioFlex® static plate was kept in the same incubator.

Microarray experiments

Preparation of RNA

For these experiments, we used fibroblasts derived from three different subjects to obtain averages of independent data and to validate generalization of the conclusions. For each of these extractions, four technical replicates were also performed to reduce the variability (Yang and Speed, 2002). Fibroblasts from each subject were grown into eight BioFlex plates to near confluence. Four plates were exposed to cyclical stretch for 96 h and the other four were kept under static conditions and used as the comparator described earlier. Total RNA was extracted from fibroblasts as previously described (Green et al., 2001). Its quantity and purity were assessed by using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and Agilent RNA 6000 Nano Assay. The ratio of 18s and 28s peaks was used to assess RNA quality. Only RNA of A260/A230 > 1.9 was used giving an Agilent trace with no evidence of RNA degradation.

cDNA microarrays

Analysis of global gene expression was carried out using cDNA microarrays containing 5866 ESTs spotted on glass (a kind gift from Dr T. Gant, MRC Toxicology Unit, Leicester). Data were deposited in accordance with Microarray Gene Expression Data Society’s MIAME recommendations in the GEO database (http://www.ncbi.nlm.nih.gov/geo; platform number GPL 4779; experiment accession numbers GSM 157871, GSM 158181 and GSM 158185; array design accession number GSE 6862). Changes in gene expression were analysed using six cDNA arrays, consisting of pairs of arrays forward and reverse labelled with Cy5 (stretched samples) and Cy3 (control samples) dyes, respectively, hybridizing RNA from stretched fibroblasts against controls. All control RNA was pooled prior to labelling to reduce variability. Microarray hybridization and analysis were performed as previously described (Turton et al., 2001; Dobbin et al., 2003). Pixel intensity for hybridization was determined using an Axon 4000A scanner and GenePix software (Axon Instruments, Union City, CA) version 3.0.6. Feature sizes were determined using the in-built automated parameters in the first instance and then adjusted manually where appropriate. The fluorescence of each pixel within the feature was determined, and the median fluorescence of these pixel measurements were taken as the measure of fluorescence for the whole feature. The local background fluorescence was measured using the default parameters of GenePix 3.0. The raw feature data for each channel were globally centered by reference to the median fluorescence of the whole feature set for that channel. The mean and SD of the log2 ratio data was calculated, and used to calculate significance weighing values.

The signs of the function and confidence values were reversed for the two tails of the distribution. Values between 0.05 and −0.05 were rounded to 0. Significance weighing values >0.92 or less than −0.92 were associated with differential expression ratios falling in the >99% confidence levels in both tails of the distribution. Thus, the middle significance weighing value of 0.5 was associated with the values falling on the 97.5% confidence level and the whole data set was thus normalized to a scale of −1 to 1.

Clustering analysis was performed using the significance weighing. Data from replicate experiments were entered separately. Data for which there was a fluorescence value in one channel only were treated as a special case. These values were assigned a significance value of +1 or −1 depending on which channel the fluorescence was recorded. Only if the intensity of that fluorescence was greater than a multiple of 0.5–3 (dependent on the array quality) it was multiplied by the average fluorescent intensity for the channel as a whole. Data processing was carried out using Convert Data version 3.2.3a (http://www.le.ac.uk/cnht/twg1/array-fp.html). These values were fed into Cluster 3 (Eisen http://www-genome.stanford.edu/ (Eisen et al., 1998) and clustered hierarchically using complete linkage. Prior to viewing, the weighed significance data were converted back into ratio data. The clustered data were displayed using Treeview (http://www.rana.lbl.gov).

Real-time PCR analysis

In order to verify the results from the microarray experiment, real-time PCR was carried out on four genes: PIP5K1C, SIPA-1, TRADD and MMP-20. An aliquot containing 1 μg of total RNA was reverse transcribed using Omniscript® Reverse Transcription kit and (QIAGEN Science, MD, USA). Real-time PCR reactions were performed using SYBR Green PCR Master Mix (QIAGEN Science), in the LightCycler® System (Roche Applied Science). Primers used were SIPAI (f− 5′- GCCCCACAGGAGTTTTTTCG; r− 5′- GGCCTCATCCGACAGAGG) , MMP2 (f− 5′- GATTAAGGAGCTACAAGCCTT; r− 5′- CCAGGGAAGGCTATAATTTG), PIP5K1C (f− 5′- TTACCTGATCCCTCGTGCAATG; r− 5′- CTTATGGTGATCGCTTTCT), TRADD (f− 5′- TTGCA-TCTCTAGCCCGAG; r− 5′- CTGACCCCGTGAAGCAGAAAGCTC) and GAPDH (f− 5′- ATGGGGGAAGGCTGAAGGTCG; r− 5′- GGTCGTATGAG GCAACAAATA).
Standard curves were generated to determine the copy number of mRNA in the experimental samples for each gene. All measurements were normalized to the expression of the GAPDH gene, the expression levels of which did not differ between stretch and non-stretch samples (data not shown).

Assessment of cytoskeleton protein morphology

Staining for cytoskeletal proteins

The protocol for triple fluorescent staining (for α-tubulin, nuclei and F-actin) of fibroblasts used has been optimized in our laboratory using commercially supplied standard reagents and following published methods (Smith, 1993; Iwig et al., 1995; Braun and Wasteneys, 1998). The fibroblasts were washed twice with phosphate-buffered saline (PBS), pH 7.4, at 37°C, and then fixed with 3.7% formaldehyde solution in PBS for 10 min at room temperature (RT). The fibroblasts were permeabilized with cold acetone at 20°C for 5 min, and then kept overnight in 1% bovine serum albumin (BSA)/PBS to reduce non-specific background staining. The wells were then incubated for 45 min at RT with monoclonal anti-α-tubulin primary antibody (clone B-5-1-2, diluted 1:2000 in 3% BSA; Sigma-Aldrich, Dorset, UK) followed by another 45 min incubation at RT with fluorescent labelled rabbit anti-mouse IgG antibody (1:200 in 3% BSA; Molecular Probes, Leiden, The Netherlands). Nuclear DNA staining was performed for 1 min using Hoechst 33 342 B2261 (1:10 000 in PBS; Sigma-Aldrich). Fibroblasts were then stained for F-actin for 20 min using Texas Red®-X Phalloidin (1:50 in PBS; Molecular Probes). The cells were washed twice for 5 min each with PBS, pH 7.4, between steps. The wells were then separated with a scalpell, and mounted on slides with cover slips.

Microscopic assessment and photography

Specimens were examined with a Nikon TE300 inverted fluorescence microscope (Nikon Corporation, Kingston-on-Thames, UK), and photographs were obtained using a Hamamatsu Orca ER black and white digital CCD camera using Openlab software (Improvision limited, Coventry, UK). The number of cells with abnormal actin morphology out of total of 300 cells was quantified by random selection of 10–20 fields per well (Hamilton, 1995). Image analysis and counting were performed by a morphologist who was blinded both to the type of treatment and to the culture condition.

Treatment regimens

Fibroblasts derived from eight hysterectomy specimens from patients without prolapse, were prepared as previously mentioned. For each source of fibroblasts, one 6-well BioFlex® plate was used for the stretch model and another one was used for the static model. The previously detailed stretch regimen was also implemented in this experiment. Each well of both plates was treated daily for 96 h with 17β-E2 10^{-8} M or 10^{-7} M (Sigma-Aldrich), levormeloxifene 10^{-6} M or 2×10^{-7} M (Novo Nordisk, Bagsvaerd, Denmark). Controls included 17α-E2 10^{-7} M (Sigma-Aldrich), or 0.1% ethanol, which was the solvent used for both E2 and levormeloxifene. The two doses of 17β-E2 used represented the low physiological and the high pharmacological doses as previously described in the literature (Liu et al., 1997; Yu et al., 1999), whereas the two doses of levormeloxifene represented the highest and lowest serum levels in women treated with this drug to prevent osteoporosis (Novo-Nordisk, 1998a).

Assessment of proliferation index

This experiment was designed to examine the effect of mechanical stretch, 17β-E2 (10^{-7} M), levormeloxifene (10^{-8} M), 0.1% ethanol or no treatment on the proliferation of cultured fibroblasts in the stretch and static models. It was performed under the same culture conditions and stretch regimen described above. Each treatment was applied to three wells in the stretch mode and another three in the static mode. The result recorded for each individual treatment represents the mean number of cells in the three wells counted four times by each of two independent observers.

Western blot analysis of ER in fibroblasts

Fibroblasts were prepared as previously described and standard western blotting techniques were used (Klinge et al., 2000; Stracke et al., 2000; Skutek et al., 2003). Briefly, fibroblasts were washed and lysed in a protein extraction buffer. A 50 μg of total protein was run on a 7.5% polyacrylamide gel (Bio-Rad, Hercules, USA), blotted and analysed by hybridization of either mouse monoclonal anti-ER-α antibody (Catalogue no. 05–394, Upstate, Charlottesville, USA) or rabbit polyclonal anti-ER-β antibody (Catalogue no. PA1-313, Affinity Bioreagents, Golden, USA).

Statistics

Confidence intervals (CIs) and P-values for the percentage of F-actin abnormalities were obtained by gamma errors regression with a log link function, while CIs and P-values for the cell counts from the proliferation experiment were obtained by Poisson regression with a log link function. All calculations were adjusted for the clustering of results from the same subjects. Calculations were performed using the glm command in Stata (StataCorp 2001. Stata Statistical Software Release 7.0. College Station, Texas: Stata Corporation).

Results

cDNA microarrays and real-time PCR

For each transcript, signal intensities were expressed as a fold change from stretch to static such that positive numbers indicate higher expression and negative numbers indicate lower expression in the stretched cells. Using our filtering methods, 16 genes were significantly up-regulated and 18 were significantly down-regulated in stretched fibroblasts compared with static fibroblasts. Table I shows a list of these 34 genes and, where known, their currently established functions. Four mechanoresponsive genes, coding for regulators of actin cytoskeleton remodelling and its interaction with the ECM, were identified. These are: Phosphatidyl inositol-4-phosphate 5-kinase (PIP5K1C), the human signal-induced proliferation associated gene-1 (SIPA-1), TNFRSF1A-associated via death domain (TRADD) and deoxyribonuclease 1-like 1 (DNase 1-L1). Two other genes that control ECM dynamics were altered: Transforming growth factor-β3 (TGF-β3) was down-regulated and matrix metalloproteinase-20 (MMP-20) was up-regulated. Differential expression of four of the genes identified in the microarray experiment was confirmed by semi-quantitative real-time PCR (Fig. 1).

Assessment of cytoskeletal morphology

Normal F-actin morphology

In normal fibroblasts, F-actin appeared as well-defined, brightly red fluorescent, discrete filaments. The stress fibres ran from one edge of the cell to the other (Fig. 2, parts 1 and 2), continued into very long filopodia (Fig. 2, part 3), and sometimes stress fibres crossed one another (Fig. 2, part 4).

Evaluation of F-actin abnormalities

A number of changes in actin phenotype following both stretching or levormeloxifene treatment were observed. The most frequent was the partial disintegration of the filaments into small spots and micro-aggregates especially at the cell periphery (Fig. 2, part 5). However, when these microfilaments aggregates coalesced, the cell shape was markedly distorted (Fig. 2, part 6). Another prominent feature in many cells was a diminished number of stress fibres (Fig. 2, parts 7–9), while an alteration of cell shape into an arborized configuration was distinctly seen in a few cells (Fig. 2, parts 10 and 11). In some cells there was extensive loss of microfilaments, particularly centrally. The fine microfilaments that remained were predominantly seen at the cell periphery (Fig. 2, parts 12 and 13).

A small number of these abnormal changes in F-actin morphology were also observed in the static specimens before any treatment. These might have been caused by the solvent (ethanol), the fixative
Table I. Mechano-responsive genes significantly altered in stretched fibroblasts and identified by cDNA microchip.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Symbol</th>
<th>GenBank accession number</th>
<th>Fold change</th>
<th>P-value 2-tail</th>
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<tr>
<td><strong>Actin remodelling regulators</strong></td>
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<tr>
<td>TNFRSF1A-associated via death domain</td>
<td>TRADD</td>
<td>NM_003789</td>
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<td>Phosphatidylinositol-4-phosphate 5-kinase, type I, γ</td>
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<td>NM_006747</td>
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<td>0.0008</td>
</tr>
<tr>
<td>Signal-induced proliferation-associated gene 1</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Deoxyribonuclease 1-like 1</td>
<td>DNase1-L1</td>
<td>NM_006730</td>
<td>1.14</td>
<td>0.049</td>
</tr>
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<td><strong>ECM/ECM dynamics regulators</strong></td>
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<tr>
<td>Matrix metalloproteinase 20 (enamelysin)</td>
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<td>Laminin, beta 2 ( laminin S)</td>
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<td>Transforming growth factor, β receptor III (betaglycan,300 kDa)</td>
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<td><strong>Cell cycle and proliferation regulator</strong></td>
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<td>0.0008</td>
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<td>E74-like factor 3 (ets domain transcription factor, epithelial-specific)</td>
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<td>Ubiquitin specific protease 13</td>
<td>USP13</td>
<td>NM_003940</td>
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<td><strong>Intracellular modulators/signal transducers</strong></td>
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<td>Electron-transfer-flavoprotein, α polypeptide (glutaric aciduria II)</td>
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<td>Mitochondrial elongation factor G2</td>
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<td>Solute carrier family 22 (organic cation transporter), member 1</td>
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<td>Tax1 (human T-cell leukaemia virus type I) binding protein 1</td>
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<td>5-lipoxygenase-activating protein</td>
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<td>Splicing factor 1</td>
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<td>Post-meiotic segregation increased 2-like 4</td>
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<td>NM_004630</td>
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<td>RER1 homolog (Saccharomyces cerevisiae)</td>
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<td>MADS box transcription enhancer factor 2, polypeptide B</td>
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<td>Carboxypeptidase A2 (pancreatic)</td>
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</table>
(formaldehyde) or the methanol used to dissolve the solid Texas Red®-X Phalloidin.

The percentage of cells with abnormal actin configuration was significantly higher in fibroblasts subjected to stretch when compared with the static model (P < 0.0001). There was a mean 5.9-fold [range 4–8; 95% CI 3.71–9.48] increase in the incidence of abnormal actin configuration in stretched versus static cells irrespective of the treatment applied. As such, separate comparisons for individual treatments were not required. In addition, the frequencies of actin abnormalities were also significantly higher with both concentrations of levormeloxifene (P < 0.0001) when compared with ethanol. This was consistent irrespective of stretch. The effect of levormeloxifene 10^{-6}M and 2 \times 10^{-7}M, with pooling of the stretch and no stretch results, was ≥2.5 times (95% CI 1.5–4.2) and ≥2.2 times (95% CI 1.5–3.3) the effect of ethanol, respectively. There were no significant differences in cell morphology between cells treated with either concentration of 17β-E2, 17α-E2 or ethanol alone. Fig. 3 shows the estimated ratios and 95% CIs for the percentage of cells with abnormal F-actin morphology. It represents the comparisons between the effect of stretch (regardless of treatment) versus no stretch, and also the comparison between the effect of individual treatment versus ethanol. The significant differences were recognized in the wells treated for ≤48 h, although marked changes started to appear after 24 h.

**Tubulin and nuclear DNA staining**

No morphological or staining differences were observed in the microtubules (Fig. 2, parts 14 and 16) or the nuclei (Fig. 2, parts 15 and 16) of cultured fibroblasts regardless of the type of treatment or the exposure to stretch.

**Time-ranging experiments**

In the experiments reported thus far, we used the maximum mechanical stretch, for the longest duration sustained by the Flexercell Stress Unit and durability of the membrane of the flex wells. We then conducted a series of time-ranging studies at 2, 4, 8, 12, 48, 72 and 96 h, where the only variable was the duration of the experiment, to detect the time at which morphological changes appear. For the 2, 4, 8 and 12 h experiments, the stretch was applied for the whole period of experimentation, whereas for the other experiments stretch was applied for 12 h alternating with the static mode. These experiments revealed that the changes in F-actin cytoskeleton started to appear after 12 h and reached a plateau after 48 h (data not shown).

In a series of initial experiments, the cells were incubated without stretch for a further four days after the 96 h of stretch. They were examined daily by light microscopy, and Phalloidin staining for F-actin was performed to assess if they were to recover. There was a gradual increased rate of cellular death and cells were detached off the membrane. By the fourth day, the living cells represented the minority that probably was not well stretched. This was the major...
Effect of stretch on fibroblast proliferation

The estimated means and 95% CI for the cell counts from the Poisson regression are shown in Table II. Stretch resulted in 23% (P = 0.04) and 28% (P < 0.001) reductions in cell count when treated with ethanol or no additive, respectively. However, treating the stretched cells with 17β-E2 10^{-9} M was associated with 39% (P = 0.02) increase in cell count. There was no significant change with levormeloxifene.

Western blots

An immunoreactive band—66 kDa—was visualized for ER-α (Fig. 4), whereas the presence of ER-β was not confirmed in fibroblasts grown as primary cultures at the sixth passage.

Discussion

We employed a cDNA microchip array to examine the change in transcriptional profile as a result of long-term application of mechanical stretch to primary cultures of fibroblasts derived from healthy cardinal ligaments, for the simultaneous analysis of a large number of genes. We used biological replicates to minimize false positive results (Claverie, 1999; Lee et al., 2000) where RNA was extracted from three different subjects, to obtain averages of independent data and to validate generalization of the conclusions. For each of these extractions, four technical replicates were also performed to reduce the within assay variability (Yang and Speed, 2002). Our filtering methods identified 34 mechano-responsive genes. Four of these genes code for regulation of actin cytoskeleton remodelling and its interaction with the ECM proteins, and were up-regulated, and two other genes that control ECM dynamics were also altered (Table I). We investigated the reliability of the data obtained by cDNA arrays by real-time PCR on four genes, and the results confirmed the pattern of differential expression in the two techniques.

Remodelling of actin cytoskeleton requires tight spatial and temporal regulation of actin filament assembly (Howe et al., 1998;
Defilippi et al., 1999; Pollard et al., 2000). Fig. 5 illustrates simplified pathways for regulation of actin assembly following cell adhesion. Cell surface interactions are transduced through small GTPases (e.g. Rho, Rac and Cdc42) (Chong et al., 1994; Hartwig et al., 1995; Pantaloni et al., 2001) which eventually lead to the conversion of PIP5K1C to phosphatidyl inositol 4,5 bisphosphate (PIP2) which, in turn, enhances F-actin assembly (Chong et al., 1994; Hartwig et al., 1995).

In contrast, the other three up-regulated actin regulatory genes identified in this study; DNase 1-L1, TRADD and SIPA-1 have negative impacts on actin cytoskeleton remodelling. The binding of DNase 1-L1 protein with G-actin prevents actin polymerization (Weber et al., 1999), and this binary complex is rendered more stable and more effective when cofilin binds to a different site on the G-actin molecule forming a ‘cofilin-actin-DNase 1-L1’ ternary complex (Chhabra et al., 2000). Signals from Rho and Rac pathways were found to inhibit the depolymerizing effect of DNase 1-L1 protein and actin depolymerising factor (ADF)/cofilin family (Edwards et al., 1999; Maekawa et al., 1999). Further, PIP2 inhibits ADF/cofilin binding to G-actin (Ojala et al., 2001) and dissociates DNase I-L1 from its complex with G-actin (Yonezawa et al., 1990).

Stimulation of TNFR-1 induces an overall decrease in F-actin and inhibits Cdc42-dependent filopodia formation in macrophages. When the TNF-induced decrease in F-actin was inhibited either pharmacologically or using receptor mutants, an increase in F-actin formation was observed in response to TNF (Peppelenbosch et al., 1999). SIPA-1 overexpression was shown in vitro to inhibit Rap1-dependent cell-matrix adhesion—the initial step in actin cytoskeleton remodelling (Tsukamoto et al., 1999). We speculate that in our model, overexpression of PIP5K1C is a compensatory response to the damage induced by DNase 1-L1, TRADD and SIPA-1. These contrasting gene profiles may be due to the long duration high amplitude of stretch used in this study. Application of mechanical stretch to human tendon fibroblasts increased the rate of apoptosis after 15 min of stretch by induction of a stress-activated protein kinase pathway (Skutek et al., 2003). The longer stretch period of 60 min was not associated with such an increase in apoptosis rate due to development of stress tolerance, possibly through heat-shock protein 72-mediated suppression of the activated stress-activated protein kinase pathway. We observed a significant reduction (P < 0.001) in cell proliferation in response to stretch. Expression of DNase I-L1 protein is increased prior to the induction of apoptosis (Polzar et al., 1994), a process that we have documented to occur when cultures were continued after cessation of stretch. However, SIPA-1 can induce cellular proliferation in lymphocytes by interacting with members of the small GTPase family (Wada et al., 1997).

Duration of stretch has been shown to have an effect on cellular proliferation in human patellar tendon fibroblasts (Zeichens et al., 2000) with cyclical stretch (1 Hz and 5% amplitude) for 15 or 60 min inducing proliferation, but not if applied for 30 min. Cyclic stretching of fetal rat lung cells (1 Hz and 5% amplitude for 48 h) significantly increased cell proliferation and reduced cell-doubling time (Liu et al., 1992). However, low magnitude cyclical stretching (0.45% at a cycle of 30 s, for 72 h) of scleral fibroblasts did not affect proliferation (Yamaoka et al., 2001). It is clear that stretch-mediated effects on cell division are influenced by the amplitude, frequency, periodicity and duration of the applied stretch as well as the cell type.

We identified many morphological abnormalities in response to stretch, notably partial disintegration of actin filaments into small spots, reduction in the number of stress fibres, alteration of the shape of many cells into an arborized configuration and dissolution of the microfilaments. Fluorescence-labelled phalloidin was used to study actin assembly because of its specificity and affinity to F-actin (Wulf et al., 1979). No cross-reaction with other cytoskeletal (including G-actin) or other cellular proteins is known (Faulstich et al., 1988). We used thinly spread whole, culture fibroblasts in our morphological assessment rather than electron microscopy due to the destructive nature of the fixatives (Fawcett and Raviola, 1994) and because the

Figure 5: Possible simplified model for regulation of actin cytoskeleton remodelling following cell adhesion

$\beta$-integrin binds to focal adhesion proteins, such as talin or $\alpha$-actinin. This induces activation of Rho family of small GTPases, which in turn influence the activity of PIP5K1C and stimulate PIP2 production. PIP2 in turn, induces actin cytoskeleton remodelling. TNFR-1 reduces F-actin possibly by inhibiting Cdc42 and/or activating ADF/Cofilin. The later is inhibited by both small GTPases and PIP5K1C. SIPA-1 negatively regulates Rap1, which is required for cell adhesion induced by ECM. DNase 1-L1 prevents actin polymerization and is inhibited by PIP2.
ultra-thin sections needed would only allow evaluation of short segments of the filaments and microtubules.

In order to ascertain that cells used in these experiments are potential targets for estrogen action, we confirmed, by western blotting, the continued expression of ER-α in fibroblasts derived from passage 6 of primary cultures. However, the mechanistic basis for SERMs-induced effects on the fibroblast cytoskeleton remain unclear. In interpreting the actin cytoskeletal changes induced by levormeloxifene, we consider the following mechanisms. First, ineffective binding of levormeloxifene for the ER may be an explanation, but our study failed to show any ameliorating effects for E2 on actin morphology. Second, levormeloxifene and/or stretch may have facilitated depolymerization of F-actin into G-actin, which was not detectable by the stain used in this experiment. Finally, levormeloxifene and/or stretch may have interfered with specific cross links of constituting proteins leading to destabilization of F-actin.

We acknowledge the differences between the paradigm of stretched fibroblasts and the in vivo conditions, including those related to deficiencies of natural mediators and cytokines, the application of arbitrary load cycles and the isolation of the fibroblasts from a potentially favourable in vivo environment. The cytoskeleton assembly is a reflection of the cell interaction with its environment and its integrity promotes cell survival. The damage observed with stretch and levormeloxifene could be an indication that this compound disturbs cellular communication with ECM. It would have been useful to identify those proteins encoded by the stretch modulated genes, but the observed cell death after stretch and the paucity of the remaining live cells precluded these experiments. The use of this experimental design represents an exciting starting point to contribute to the understanding of mechanisms of drug actions on fibroblasts.

Acknowledgement

We would like to acknowledge Mrs Carol Orme for the excellent technical help in conducting the stretch experiment, Professor Brian Williams for his support, Dr A. Green for technical advice and Dr. May Elnawawy for her help in producing the figures.

Funding

The project was supported by the funds of the Gynaecology Research Unit, University Hospital of Leicester.

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Submitted on September 27, 2007; resubmitted on November 5, 2007; accepted on November 26, 2007